Nature Methods

High-frequency genome editing using ssDNA oligonucleotides with zinc-finger nucleases

Fuqiang Chen, Shondra M Pruett-Miller, Yuping Huang, Monika Gjoka, Katarzyna Duda, Jack Taunton, Trevor N Collingwood, Morten Frodin & Gregory D Davis

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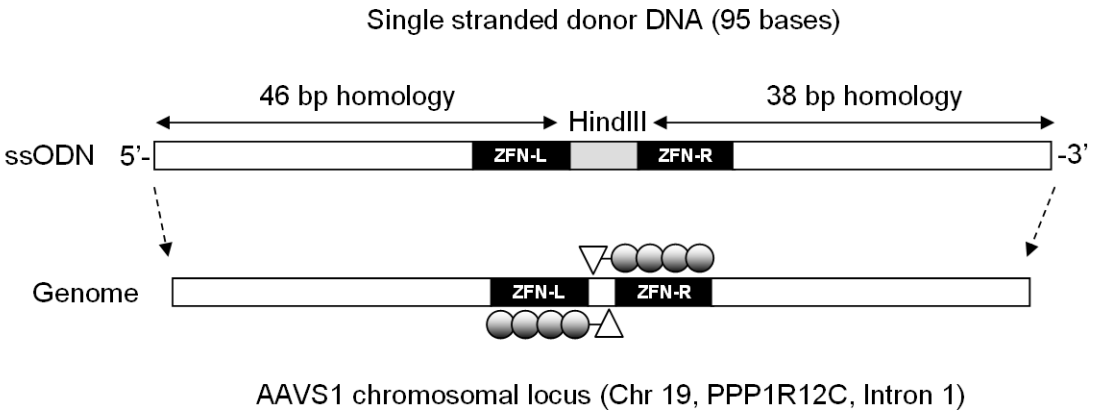
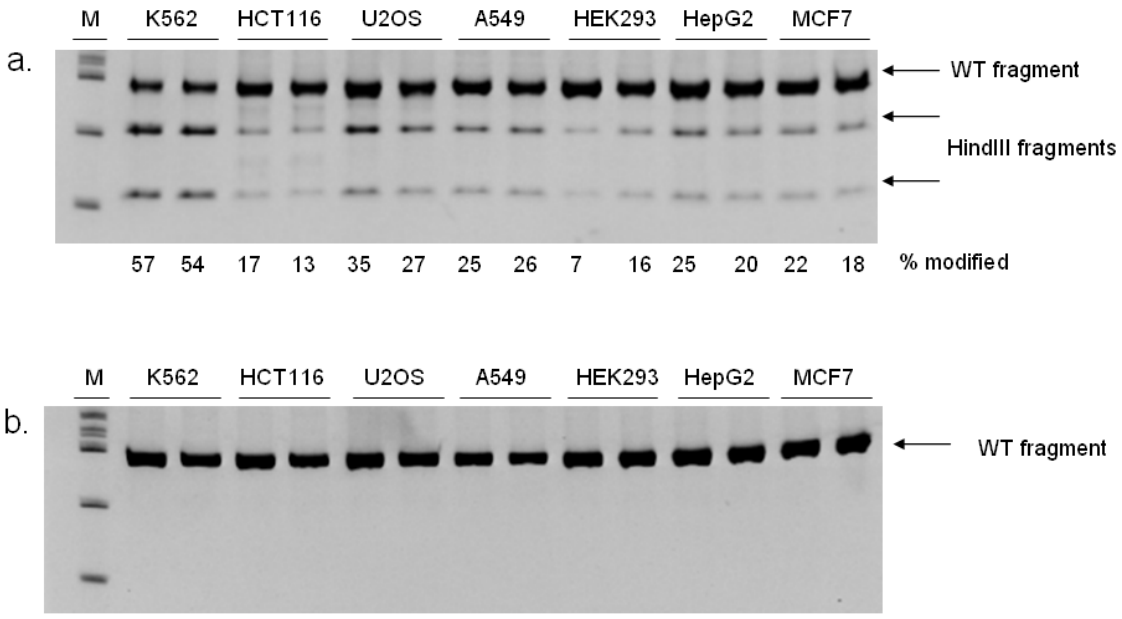


Supplementary Figure 1. Structure of the ssODN used to modify the AAVS1 locus in different cell types. A 95-mer ssODN (AAVS1-95) was used to incorporate a HindIII site into the AAVS1 locus. ssODNs were made using standard phosphoramidites (e.g. no chemical modifications) and PAGE purified. The DNA sequence of AAVS1-95 is shown in Supplementary Note 1.



Supplementary Figure 2. Frequency of ssODN-based ZFN mutagenesis in various transformed cell types at the AAVS1 locus. A 95-mer ssODN (AAVS1-95) was used to incorporate a HindIII site into the AAVS1 locus. Cells were nucleofected and genomic DNA was harvested 2 days post nucleofection. Genomic DNA was PCR amplified, digested with HindIII, and resolved on a 10% acrylamide gel. The proportion of cleaved DNA was quantified by densitometry. (**a**) Each cell type was transfected with 0.3 nmol of ssODN and 4 µg (left lane) or 8 µg (right lane) of ZFN mRNA. (**b**) In the absence of ZFN mRNA, each cell type was transfected with 0.3 nmol of ssODN (left lane) or 2.5 µg of a GFP plasmid (right lane). M: DNA marker.

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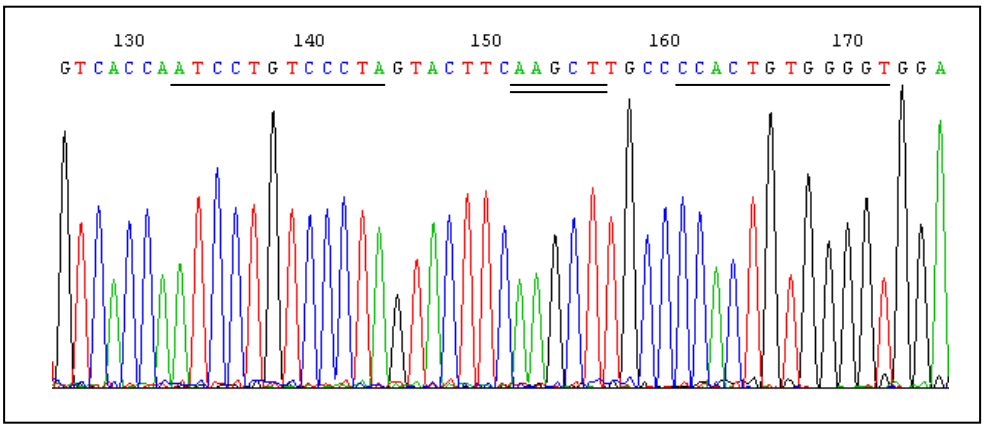

Supplementary Figure 3. DNA sequence of an A549 clone (P2F11) modified at the AAVS1 locus. DNA sequencing confirms incorporation of an ssODN-harbored HindIII site at the targeted position in the AAVS1 locus. A549 cells were nucleofected with 0.1 nmol of a 115-mer ssODN (AAVS1-115) along with 8 µg of AAVS1 ZFN mRNA. Clones were selected by SYBR Green real-time PCR (see on-line Methods) and HindIII digestion assays, and confirmed by DNA sequencing. The HindIII site is double underlined. The ZFN binding sites are underlined. The sequence is in the anti-sense strand orientation.

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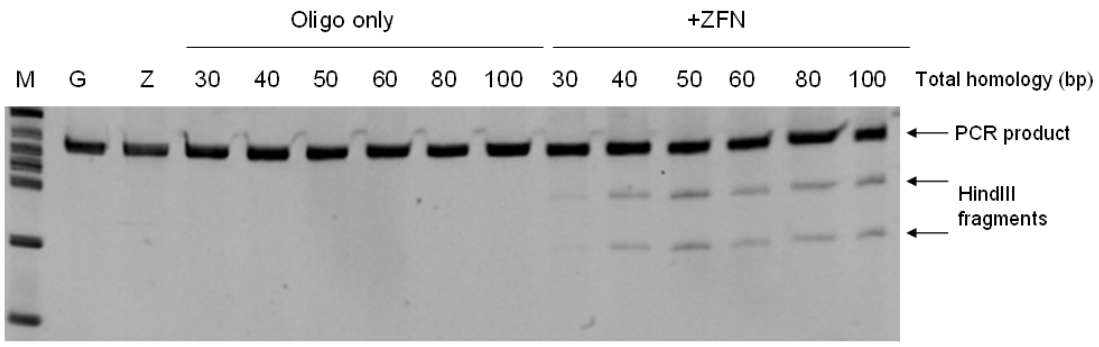
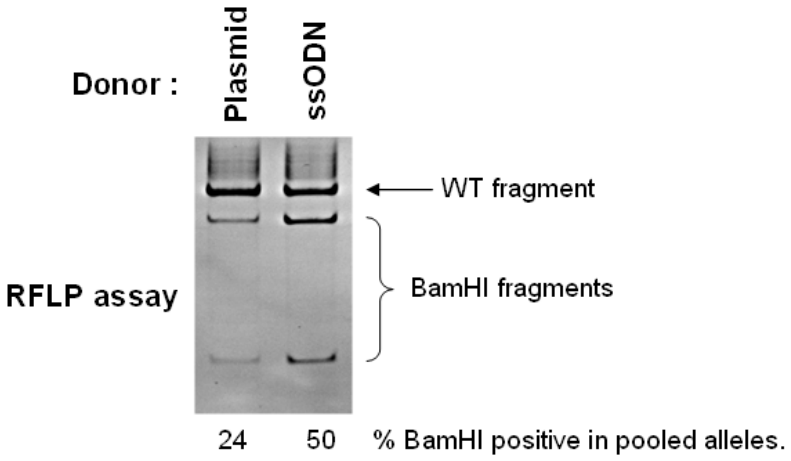
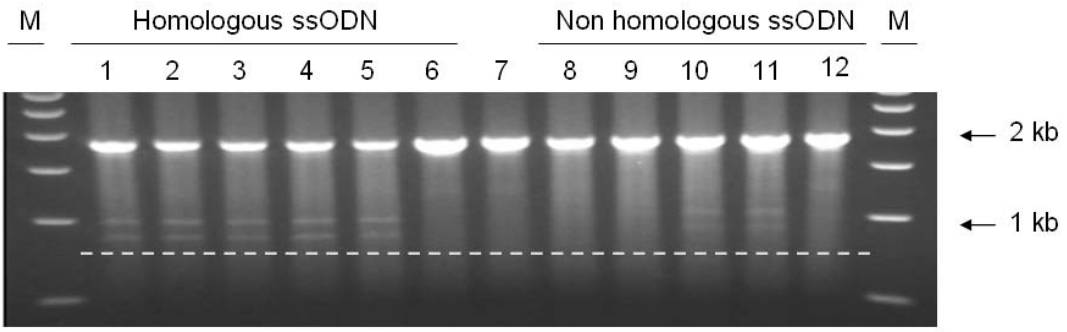


Supplementary Figure 4. Effect of ssODN homology length on HindIII site insertion efficiency at the AAVS1 locus. Numbers represent the total homology length of the ssODN donor. For example, a 30-mer donor has two 15-base homology arms. Each ssODN contained a HindIII site between the homology arms. The DNA sequence of each ssODN is shown in Supplementary Note 1. K562 cells were nucleofected with 2.5 µg of plasmid DNA for each ZFN (5 µg total) and 0.3 nmol of ssODN. Cells were harvested 2 days post nucleofection. Genomic DNA was PCR amplified and digested with HindIII. M: Marker. G: GFP. Z: ZFN.

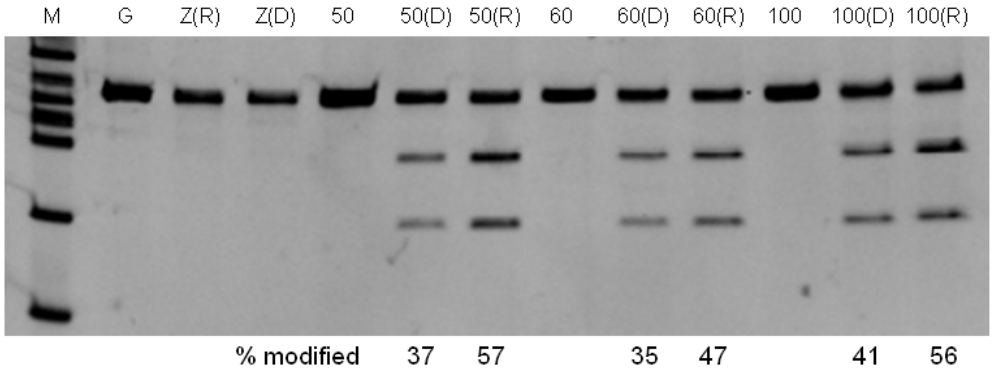


Supplementary Figure 5. Comparison of ZFN-mediated point mutation frequencies at the RSK4 locus (alias RPS6KA6) using ssODN versus plasmid donors. A plasmid or an ssODN donor with homology of 1,565 bp and 109 bp, respectively, were used to incorporate a codon switch and a diagnostic BamHI site into the RSK4 locus (Supplementary Note 1). The ssODNs (RSK4-114) were made using standard ssODN synthesis (e.g. no chemical modifications) and PAGE purified. K562 cells were nucleofected with 10 µg of plasmid or 0.2 nmol of ssODN together with 4 µg of ZFN mRNA and genomic DNA was harvested 3 days post nucleofection. Genomic DNA was PCR amplified, digested with BamHI and resolved on a 6% acrylamide gel. The proportion of cleaved DNA in was quantified by densitometry.

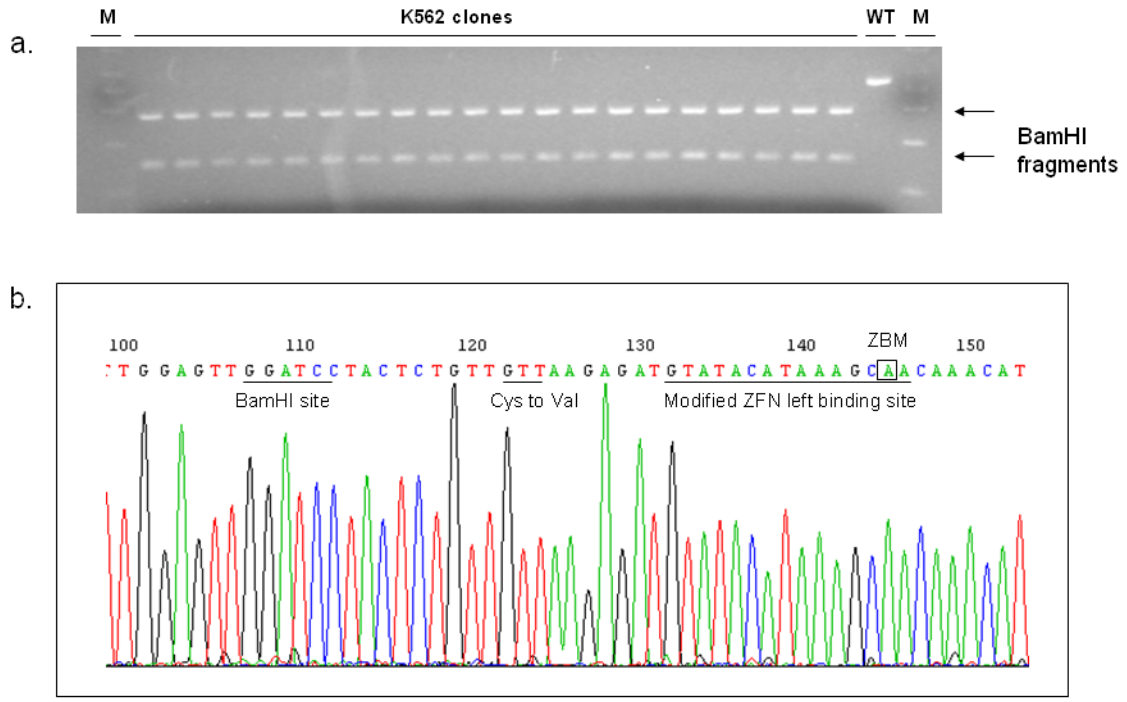


Supplementary Figure 6. Homologous versus non-homologous ssODN-mediated modification at the AAVS1 locus in A549 cells. Both ssODNs (homologous and non-homologous) were of the same length and carried a HindIII site on the center. The non-homologous ssODN (CNR1-115) contained homology arms corresponding to a ZFN target site in the CNR1 locus. 1&8: sense single stranded; 2&9: antisense single stranded; 3&10: sense plus antisense; 4: sense single stranded (2X); 5&11: double stranded (pre-annealed); 6&12: sense plus antisense ssODNs only (no ZFN); 7: GFP, M: DNA marker. The dotted line is aimed to help show that cleaved bands in lanes 10 and 11 are larger than those of lanes 1-5 (also visible via relative location to 1 kb marker). Cells were nucleofected with 8 µg of AAVS1 ZFN mRNA and 0.1 nmol of homologous ssODN (AAVS1-115) or non-homologous ssODN (CNR1-115), and harvested 2 days post nucleofection. Genomic DNA was PCR amplified and digested with HindIII and resolved on 1% agarose gel.

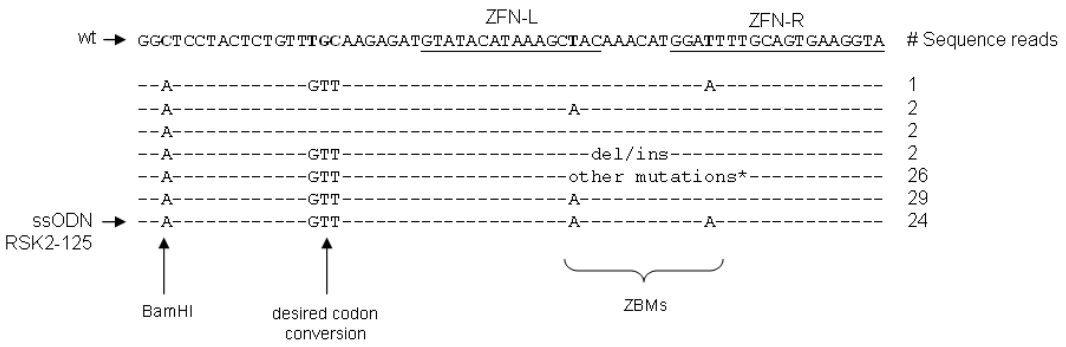
Identical results were obtained using both the sense and antisense versions of ssODN AAVS1-115 (lanes 1 & 2) and of ssODNs AAVS1-30H through AAVS1-100H (Supplementary Note 1, data not shown). Integration of a non-homologous ssODN (CNR1-115) at the AAVS1 locus was not detected (lanes 8 & 9). However, integration of a non-homologous double stranded ODN (dsODN) was detectable when both sense and antisense ssODNs were either co-transfected directly or when pre-annealed prior to transfection. The slightly larger size of the cleaved bands following non-homologous dsODN integration (lanes 10 & 11) is consistent with non-homologous or microhomology-driven end capture (i.e. insertion) of the entire ODN duplex, which has been observed in previous independent experiments1. This suggests that the single-stranded format of ODNs results in fewer (if any) non-faithful integrations in ZFN-mediated genome editing applications.



Supplementary Figure 7. Integration of ssODN donors at the AAVS1 locus when ZFNs are delivered as mRNA or plasmid DNA. Cells were nucleofected with 2.5μg DNA or 2.0 μg of mRNA of each ZFN (5 μg or 4 μg total, respectively) and 0.3 nmol of ssODN. Each ssODN contained a HindIII site between the homology arms. Cells were harvested 2 days post nucleofection. Genomic DNA was PCR amplified and digested with HindIII. M denotes marker. G denotes GFP control plasmid (no ZFN). Z denotes ZFN. (R) and (D) denote mRNA and plasmid DNA, respectively. Numbers at the top represent the total homology length of the ssODN donor. For example, a 50 bp donor has 25 bp homology arms flanking the HindIII site. Numbers at the bottom are quantification by densitometry of the frequency of targeted insertion of the HindIII site.

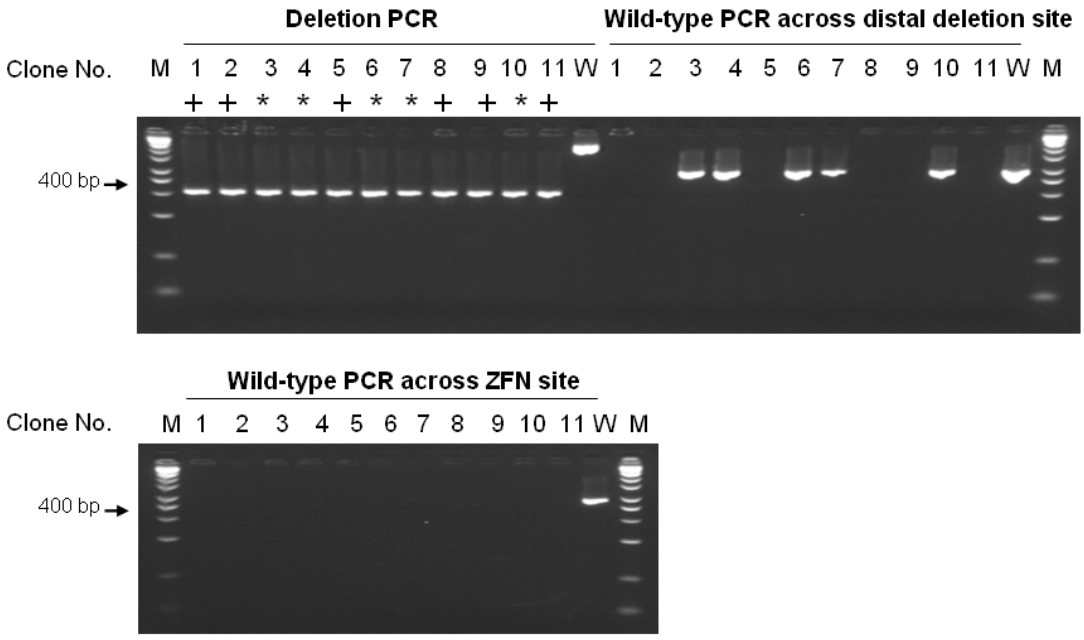


Supplementary Figure 8. Isolation of single cell K562 clones harboring ssODN-derived mutations at the RSK2 locus. (**a**) Approximately 750 single cell clones were screened using a SYBR Green qPCR assay (see on-line Methods) to identify 45 candidate clones harboring a biallelic BamHI site and nearby Cys to Val conversion. Positive clones were subsequently PCR amplified around the target locus and subjected to direct sequencing without cloning. Of the 45 candidate clones, 17 failed sequencing, 6 contained unwanted mutations in addition to the targeted mutation. Twenty of the sequence-verified clones were expanded and further validated by BamHI digestion and resolving on a 3% agarose gel. WT: Wild type; M: DNA marker. (**b**) DNA sequence of a representative clone derived using ssODN RSK2-125. DNA sequencing confirms incorporation of ssODN-harbored mutations.

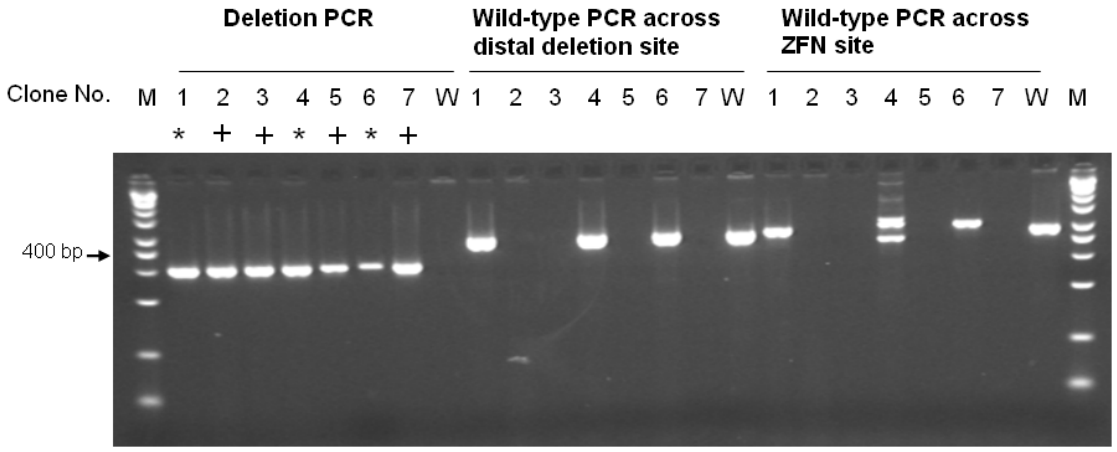


Supplementary Figure 9. Frequencies of intended and unintended mutations at the RSK2 locus following modification by ZFNs and ssODN RSK2-125. Following nucleofection with ZFN mRNA for RSK2 and ssODN RSK2-125, alleles containing the BamHI conversion were preferentially amplified by PCR, cloned, and sequenced. *Among the 26 alleles that contained “other mutations”, 8 had a point mutation outside the ssODN RSK2-125 sequence; 3 had a deletion or point mutation at the Cys to Val codon conversion site; 1 had a point mutation at the BamHI site; and the remainder had deletions or point mutations elsewhere within the ssODN sequence.

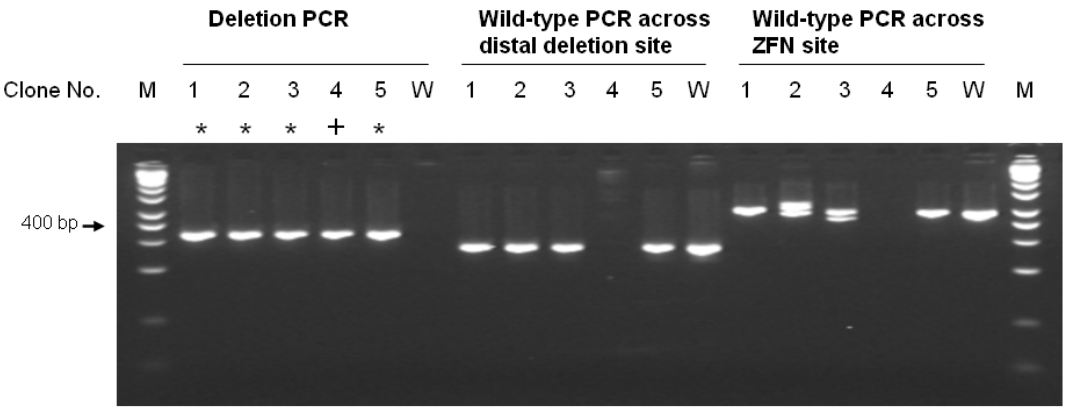
For alleles containing the BamHI conversion, the most frequent alleles also contain the desired Cys436Val conversion (82/86 alleles, 95%). Of these, 56 alleles (65% of total) contain one or both ZBMs (in addition to BamHI and Cys436Val) but no other mutations. This result suggests predominantly directional incorporation of the ssODN in a manner consistent with previous plasmid-based DSB-stimulated homologous recombination. Additionally a significant fraction of BamHI positive alleles (29/86, 34%) lack the ZBM present on the opposite side of the ZFN cut site, suggesting a unidirectional incorporation initiated from only one side or the other (but not both simultaneously) of the ZFN-induced DSB. Moreover, the fidelity of this approach is sufficient to ensure isolation of correctly targeted mutant clones even when the ZFNs cleave at a distance from the mutation target site.



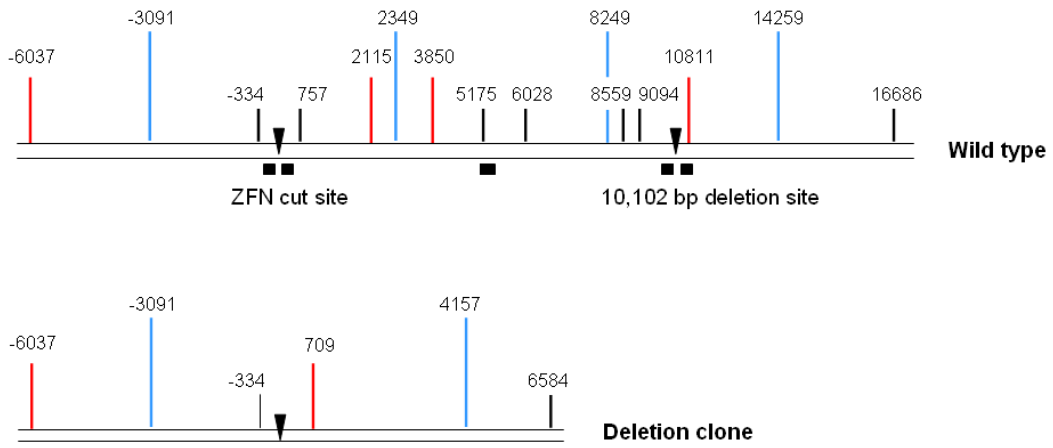
Supplementary Figure 10. PCR analysis of isolated single cell clones harboring a 1 kb genomic DNA deletion at the AAVS1 locus in K562 cells. Single cell clones were first screened using a deletion PCR assay to detect the presence of a deletion allele. Candidate clones were then analyzed by a wild-type PCR assay across both the distal border of the deleted region and across the ZFN cut site (see on-line Methods) to detect the presence of either a wild type allele or a partial deletion allele. PCR products were resolved on 3% agarose gel. Clones marked by a plus sign (+) were identified as homozygous deletions; and clones marked by an asterisk (*) were identified as heterozygous deletions. M = DNA marker; W = control wild-type cells.



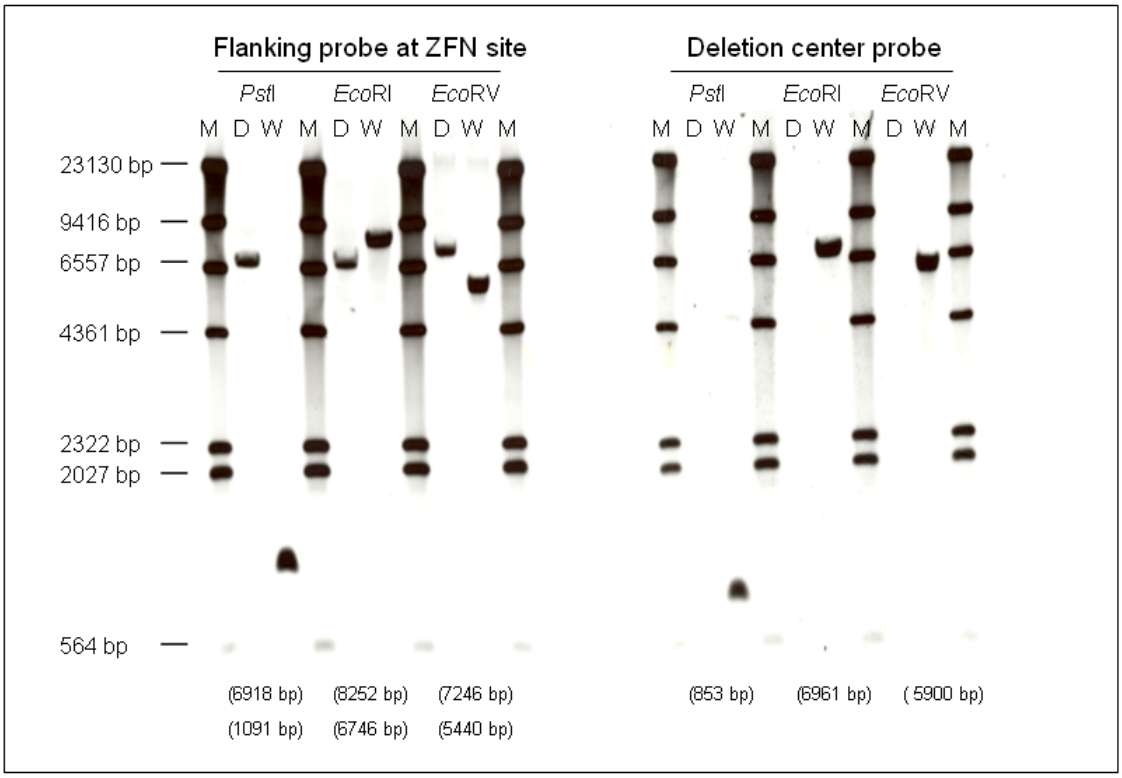
Supplementary Figure 11. PCR analysis of isolated single cell clones harboring a 5 kb genomic DNA deletion at the AAVS1 locus in K562 cells. Single cell clones were first screened using a SYBR Green qPCR assay and candidate clones were then analyzed by a deletion PCR assay to detect the presence of a deletion allele and with a wild-type PCR assay across both the distal border of the deletion and across the ZFN cut site to detect the presence of either a wild type allele or a partial deletion allele (see on-line Methods). PCR products were resolved on 3% agarose gel. Clones marked by a plus sign (+) were identified as homozygous deletions; and clones marked by an asterisk (*) were identified as heterozygous deletions. M = DNA marker; W = control wild-type cells.



Supplementary Figure 12. PCR analysis of isolated single cell clones harboring a 10 kb genomic DNA deletion at the AAVS1 locus in K562 cells. Single cell clones were first screened using a SYBR Green qPCR assay and candidate clones were then analyzed by a deletion PCR assay to detect the presence of a deletion allele and with a wild-type PCR assay across both the distal border of the deletion site and across the ZFN cut site to detect the presence of either a wild type allele or a partial deletion allele (see on-line Methods). PCR products were resolved on 3% agarose gel. Clones marked by a plus sign (+) were identified as homozygous deletions; and clones marked by an asterisk (*) were identified as heterozygous deletions. M = DNA marker; W = control wild-type cells.



Supplementary Figure 13. Restriction maps of the AAVS1 locus and an ssODN-specified 10 kb deletion at the locus mediated by an AAVS1 ZFN. The wild type restriction map was constructed based on the human Reference Sequence: NT_011109.16. The deletion restriction map was based on the predicted deletion as specified by the ssODN sequence. Black vertical lines denote PstI restriction sites; red vertical lines denote EcoRI restriction sites; and blue vertical lines denote EcoRV restriction sites. The number above each vertical line denotes the distance from the ZFN cut site. Solid rectangles represent Southern hybridization probes used in Supplementary Figure 14. Probes at the 10 kb deletion site target repetitive sequences and produced no discrete banding patterns.



Supplementary Figure 14. Southern blot analysis of a K562 clone with an ssODN-specified 10 kb deletion at the AAVS1 locus mediated by an AAVS1 ZFN. Genomic DNA (15 µg each digestion) was digested with *Pst*I, *Eco*RI, and *Eco*RV. Digested DNA was resolved on a 0.7% agarose gel, transferred to a nylon membrane, and hybridized with DIG-labeled DNA probes. The flanking probe at the ZFN site is immediately adjacent to the deletion junction, and the deletion center probe is located 5 kb from the ZFN site (see Supplementary Figure 13). M: DIG labeled molecular marker; D: 10 kb deletion clone; W: wild type. Numbers within the parentheses denote the predicted restriction fragment length for the 10Kb deletion (upper) and wild type (lower) locus, as specified by the ssODN.

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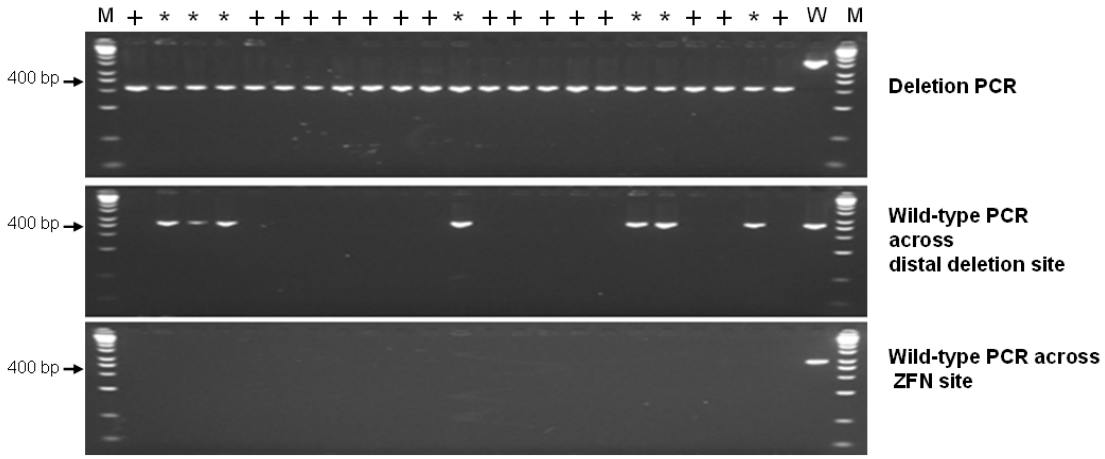

Supplementary Figure 15. Analysis of isolated single cell clones harboring a 500 bp genomic DNA deletion at the AAVS1 locus in K562 cells. Single cell clones were first screened using a deletion PCR assay to detect the presence of a deletion allele and candidate clones were then analyzed by wild-type PCR assay across both the distal border of the deletion and across the ZFN cut site to detect the presence of either a wild type allele or a partial deletion allele (see on-line Methods). PCR products were resolved on 3% agarose gel. Clones marked by a plus sign (+) were identified as homozygous deletions; and clones marked by an asterisk (*) were identified as heterozygous deletions. M = DNA marker; W = control wild-type cells.

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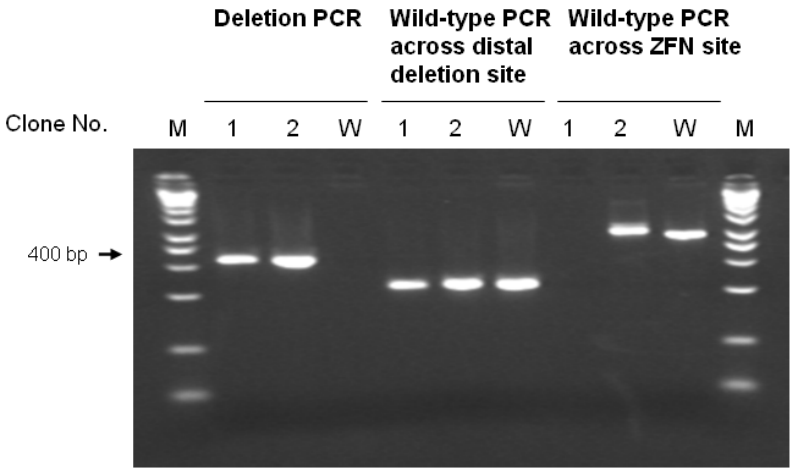
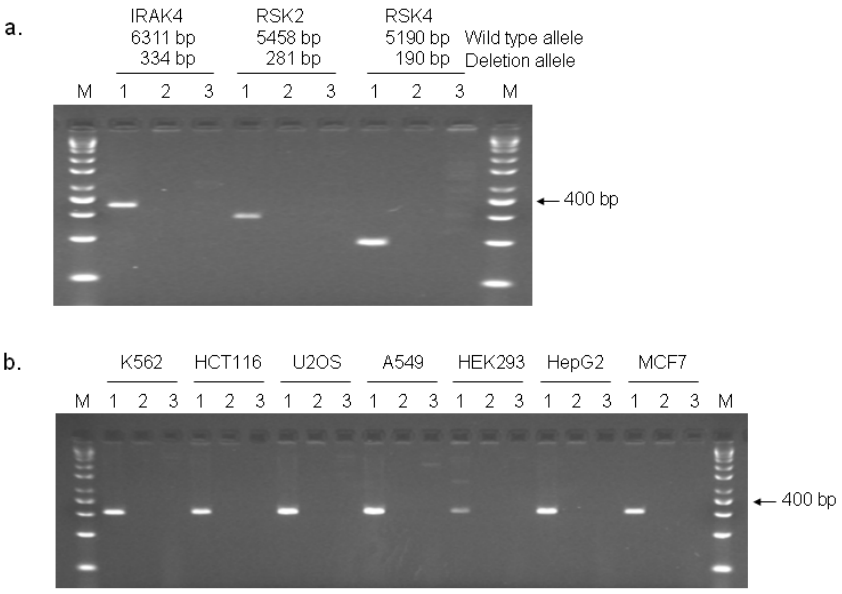
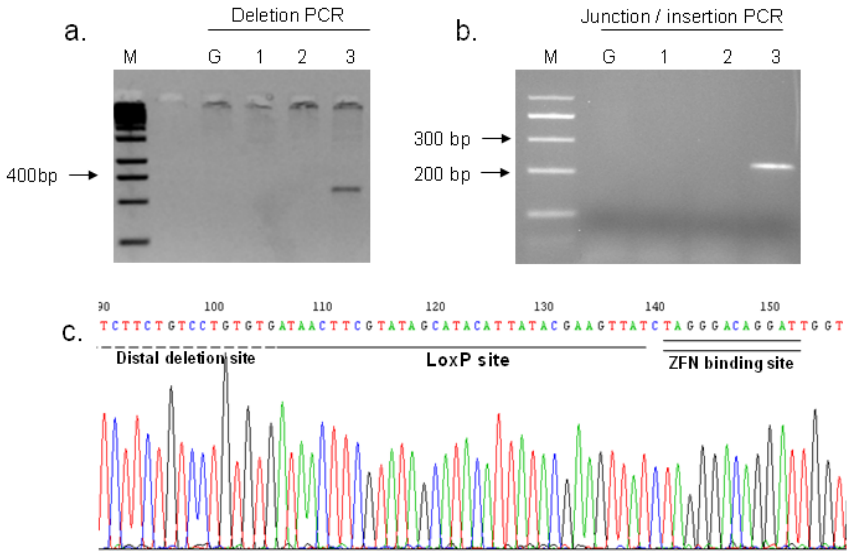


Supplementary Figure 16. PCR analysis of isolated single cell clones harboring a 100 kb genomic DNA deletion at the AAVS1 locus in K562 cells. Single cell clones were first screened using a SYBR Green qPCR assay and candidate clones were then analyzed by a deletion PCR assay to detect the presence of a deletion allele and with a wild-type PCR assay across both the distal border of deletion and across the ZFN cut site to detect the presence of either a wild type allele or a partial deletion allele (see on-line Methods). PCR products were resolved on 3% agarose gel. Both clones 1 and 2 were identified as heterozygous deletions. M = DNA marker; W = control wild-type cells.



Supplementary Figure 17. Targeted genomic DNA deletion across multiple cell types and loci. (**a**) Approximately $0.5x10^6$ K562 cells were nucleofected with 8 μg of corresponding ZFN mRNA and 0.3 nmol of corresponding ssODN (see Supplementary Note 3). Cells were harvested at 2 days post-nucleofection and genomic DNA was extracted and then PCR amplified. PCR products were resolved on 3% agarose gel. The expected PCR fragment sizes of the wild type and deletion alleles are indicated. The large wild type PCR fragments were not detectable under the experimental conditions optimized to detect the shorter deletion PCR product. The deletion PCR fragments were verified by DNA sequencing. 1 = ssODN + ZFN; 2 = ssODN only; 3 = ZFN only; M = DNA marker. (**b**) Approximately $0.5x10^6$ (K562) or $0.6x10^6$ (HCT116, U2OS, A549, HEK293, and HepG2) or $1.2x10^6$ (MCF7) cells were nucleofected with 8 μg ZFN mRNA and 0.3 nmol of ssODN (AAVS1 5' 5 kb deletion ssODN; see Supplementary Note 2). Cells were harvested at 2 days post-nucleofection and genomic DNA was extracted and then PCR amplified. PCR products were resolved on 3% agarose gel. The wild type PCR fragment is 5,303 bp and was not detectable under the PCR conditions optimized to detect the smaller deletion product. The expected deletion PCR fragment is 303 bp. The deletion PCR fragment was verified by DNA sequencing. 1 = ssODN + ZFN; 2 = ssODN only; 3 = ZFN only; M = DNA marker.



Supplementary Figure 18. Targeted 5 kb genomic DNA deletion at the AAVS1 locus and concerted insertion of 34 bp loxP site. Approximately 1.0x10^6 K562 cells were nucleofected with 4 μg ZFN mRNA and 0.3 nmol of ssODN (AAVS1 5' 5 kb deletion + loxP insertion ssODN; see Supplementary Note 3). Cells were harvested at 2 days post-nucleofection, genomic DNA was extracted and PCR amplified. PCR products were resolved on 3% agarose gel. 1 = ZFN only; 2 = ssODN only; 3 = ZFN + ssODN; M = DNA marker, G= GFP transfection efficiency control. (**a**) The wild-type PCR fragment is 5,303 bp and was not detectable under the PCR conditions optimized to detect the deletion product. The expected deletion PCR fragment is 337 bp. (**b**) The expected junction / insertion PCR fragment is 230 bp. (**c**) The deletion PCR fragment was verified by DNA sequencing.

Supplementary Table 1. Derivation of clonal cell isolates harboring ssODN mediated genomic deletions at the AAVS1 locus. Approximately $0.5x10^6$ K562 cells were nucleofected with 8 μg ZFN mRNA and 0.3 nmol of corresponding ssODN (see Supplementary Note 2 for DNA sequences). Single cell-derived clones were obtained by flow cytometry. For 100 bp to 1 kb deletions, clones were screened using a deletion PCR assay; for 5 kb to 100 kb deletions, clones were first screened using a SYBR Green qPCR assay. Candidates for 500 bp to 100 kb deletions were further analyzed by deletion PCR assay and with a wild-type PCR assay across both the distal border of the deletion and across the ZFN cut site (see On-line Methods). PCR products from homozygous clones were subjected to DNA sequencing directly without cloning. All homozygous clones were verified to harbor the expected deletions.

Targeted deletion size	Deletion ssODN	No. of clones screened	No. of homozygous clones	No. of heterozygous clones
100 bp (5')	d5-AAVS1-0.1kb	207	36 (17.4%)	57 (27.5%)
100 bp (3')	d3-AAVS1-0.1kb	256	53 (20.7%)	50 (19.5%)
500 bp	d5-AAVS1-0.5kb	288	31 (10.8%)	76 (26.4%)
1 kb	d5-AAVS1-1kb	204	6 (2.9%)	32 (15.7%)
5 kb	d5-AAVS1-5kb	620	4 (0.6%)	19 (3.1%)
10 kb	d5-AAVS1-10.1kb	630	1 (0.2%)	41 (6.5%)
100 kb	d5-AAVS1-100kb	1180	0	2 (0.2%)

Supplementary Table 2. PCR primer sequences (5'-3').

AAVS1-F1	CATGGCATCTTCCAGGGGT
AAVS1-R1	GGAATCTGCCTAACAGGAGGTG
AAVS1-F2	GGCCCTGGCCATTGTCACTT
AAVS1-R2	GGAACGGGGCTCAGTCTG
AAVS1-F3	TTCGGGTCACCTCTCACTCC
AAVS1-R3	GGCTCCATCGTAAGCAAACC
RSK2-F1	GCATGCTGAGTAACATACTTCCCTA
RSK2-R1	GAGGTGTAAACTGCTACTGCTCTG
RSK4-F1	TTGCCTGTGGGTATCAAGAAAG
RSK4-R1	TGATAGCACTACAGAATAATAATTGGTGT
AAVS1-d5R-com	GGCTCCATCGTAAGCAAACC
AAVS1-d5F-0.1	TTCGGGTCACCTCTCACTCC
AAVS1-d5F-0.5	CTTTCCGGAGCACTTCCTTC
AAVS1-d5F-1	GACCCATGCAGTCCTCCTTAC
AAVS1-d5F-1.5	GAGGCGCGTCTGATGCT
AAVS1-d5F-2	TCATCTGGCGATTTCCACTG
AAVS1-d5F-2.5	AGGACAAGTAGTGCACAGAAGACA
AAVS1-d5F-3	ACCAATCCCAGCATGCCTT
AAVS1-d5F-3.5	AAGTGAAGGAGAAAGCGGCTG
AAVS1-d5F-4	CAAATGAGCAGGGTCGCAGT
AAVS1-d5F-4.5	CGGATGAAGACCCCATCTTG
AAVS1-d5F-5	GTTCCCACTTCTTCTGTAACAGCA
AAVS1-d5F-10	AGGCAAGACAATGTGGTTGATAAG
AAVS1-d5F-20	CTCCTTCCTGTGCACATGGTTC
AAVS1-d5F-50	GGAGGCAGAAGCTGGGAG
AAVS1-d5F-100	CAGATGAAGGGATATTGCCTGAAG
AAVS1-d5loxP-5	TCTTCTGTCCTGTGTGataacttc
AAVS1-d3F-com	TTCGGGTCACCTCTCACTCC
AAVS1-d3R-0.1	GGCTCCATCGTAAGCAAACC
AAVS1-d3R-2	GGAGCTGAGGAAGGAGTGAAG
IRAK4-d5F-5.9	AGACCAACCTGTAGAAACTGGAATG
IRAK4-d5R-5.9	TGAATCGTGAATAGAGTGACAGCA
RSK2-d3F-5.2	CTAAAGGCATTATGGGACTCTTCAC
RSK2-d3R-5.2	GGTCAGTAGGATAATTCAGCTAATAGCAG
RSK4-d3F-5	GGAAATGCTGCACAATTTGGT
RSK4-d3R-5	CCCCAACTTCACTTTCACTGCT
AAVS1-C-F1	CACAGTGGGGCAAGCTTGA
AAVS1-C-R1	GGAATCTGCCTAACAGGAGGTG
AAVS1-C-F2	ACAGTGGGGCCACTAGGGA
RSK2-Bam-F1	GAAGTAAAAGAAGATATTGGAGTTGGA
RSK2-wt-F1	GAAGTAAAAGAAGATATTGGAGTTGGC
RSK2-Bam-R1	CTGGTACTGACATGCATGAACAAG
RSK2-Val-F	GGAGTTGGATCCTACTCTGTTGTT
RSK2-Cys-F	GGAGTTGGCTCCTACTCTGTTTG
AAVS1-d5R-C	GGAATCTGCCTAACAGGAGGTG
AAVS1-d5F-0.5wt	CTTTCCGGAGCACTTCCTTC
AAVS1-d5R-0.5wt	GGCTGGAAGAGCTAGCACAG
AAVS1-d5F-1wt	GACCCATGCAGTCCTCCTTAC
AAVS1-d5R-1wt	AGCAACACAGCAGAGAGCAAG
AAVS1-d5F-5wt	GTTCCCACTTCTTCTGTAACAGCA
AAVS1-d5R-5wt	CCCCACACTTTCCACATACTTACAC

Supplementary Table 2 continued. PCR primer sequences (5'-3').

AAVS1-d5F-10wt	AGGCAAGACAATGTGGTTGATAAG
AAVS1-d5R-10wt	AGGAGAATGGCGTGAACCTG
AAVS1-d5F-100wt	CAGATGAAGGGATATTGCCTGAAG
AAVS1-d5R-100wt	AGATACAGTTGGCCACTCCCTC
AAVS1-Z-CF	TTCGGGTCACCTCTCACTCC
AAVS1-Z-CR	GGCTCCATCGTAAGCAAACC
AAVS1-S-F1	CTAGGGACAGGATTGGTGACAG
AAVS1-S-R1	ACCTAGGACGCACCATTCTCAC
AAVS1-S-F2	CTAGGGACAGGATTGGTGACAG
AAVS1-S-R2	CAGCACCAGGATCAGTGAAAC
AAVS1-PZ-F1	CTTTCCGGAGCACTTCCTTC
AAVS1-PZ-R1	GGGGTGGAGGGGACAGA
AAVS1-PZ-F2	CTGCACCACGTGATGTCCTC
AAVS1-PZ-R2	AAAGTACCCAGAACCAGAGCCAC
AAVS1-Pd5-F1	TGGATTTGACAGTCCTCAGATTG
AAVS1-Pd5-R1	CCCAGGCTGGATTGCAC
AAVS1-Pd5-F2	CCTTGTCTTGGCCAATTTCTAGC
AAVS1-Pd5-R2	CTCTCCACACAGGACAGAAGAGTC
AAVS1-Pd10-F1	GAGATGGAGTCTTGCTCTGTCG
AAVS1-Pd10-R1	AACTCTGGTGCCAACTTGGAC
AAVS1-Pd10-F2	CAGGTTCACGCCATTCTCCT
AAVS1-Pd10-R2	CTGCTTCCCTGATCATTCCTG
AAVS1-Pwt10-F1	GGACAGCTAGTGTGGGAGCAG
AAVS1-Pwt10-R1	ACAACTCCCCTATCCTACCCATC
AAVS1-Pwt10-F2	GTTGCCCAGGCTGGAGTG
AAVS1-Pwt10-R2	TGGTGCCAAACCCGTATACTC

Supplementary Note 1. DNA sequences for ssODNs used to create small insertions, codon changes, and point mutations. ZFN binding sites are underlined. Insertions are uppercase bold. Codon changes and point mutations are lowercase bold. For ssODNs AAVS1-30H, -40H, -50H, -60H, -80H, and -100H, the number at the end of each AAVS1 ssODN donor name denotes the total homology length of the ssODN. For the remaining ssODNs the number indicates to total ssODN length.

>AAVS1-95
CTGGTTCTGGGTACTTTTATCTGTCCCCTCCACCCCACAGTGGGGC**AAGCTTGAAGT**ACTAGGGACAGGATTGGTG
ACAGAAAAGCCCCATCCTT

>AAVS1-115
GTGGCTCTGGTTCTGGGTACTTTTATCTGTCCCCTCCACCCCACAGTGGGGC**AAGCTTGAAGT**ACTAGGGACAGGA
TTGGTGACAGAAAAGCCCCATCCTTAGGCCTCCTCCTTC

>AAVS1-30H
ACCCCACAGTGGGGC**AAGCTT**CACTAGGGACAGGAT

>AAVS1-40H
CCTCCACCCCACAGTGGGGC**AAGCTT**CACTAGGGACAGGATTGGTG

>AAVS1-50H
TGTCCCCTCCACCCCACAGTGGGGC**AAGCTT**CACTAGGGACAGGATTGGTGACAGA

>AAVS1-60H
TTATCTGTCCCCTCCACCCCACAGTGGGGC**AAGCTT**CACTAGGGACAGGATTGGTGACAGAAAAGC

>AAVS1-80H
CTGGGTACTTTTATCTGTCCCCTCCACCCCACAGTGGGGC**AAGCTT**CACTAGGGACAGGATTGGTGACAGAAAAGC
CCCATCCTTA

>AAVS1-100H
GGCTCTGGTTCTGGGTACTTTTATCTGTCCCCTCCACCCCACAGTGGGGC**AAGCTT**CACTAGGGACAGGATTGGTG
ACAGAAAAGCCCCATCCTTAGGCCTCCTCC

>CNR1-115
CCGCAGCCTCCGCTGCAGGCCTTCCTACCACTTCATCGGCAGCCTGGCGGTG**AAGCTTGAA**GCAGACCTCCTGGGG
AGTGTCATTTTTGTCTACAGCTTCATTGACTTCCACGTG

>RSK2-125
GGATATGAAGTAAAAGAAGATATTGGAGTTGG**a**TCCTACTCTGTT**gtt**AAGAGATGTATACATAAAGC**a**ACAAACA
TGGA**a**TTTGCAGTGAAGGTAAATTTTTTTTATTTAAAATGCAATTCATA

>RSK4-114
TTGGTGAAGTATATGAATTGAAGGAGGATATTGGTGTTGG**a**TCCTACTCTGTT**gtt**AAGCGATGCATACATGC**t**AC
TACCAACATGGAATTTGCAGTGAAGGTATTGTCTCTGA

Supplementary Note 2. DNA sequences for ssODNs used to create deletions at the AAVS1 locus. Distal deletion sites are lowercase. The right or left ZFN binding sites are underlined. d5 indicates a deletion 5' to the ZFN cut site (in the upstream direction on the sense strand of the gene sequence) and d3 indicates a deletion 3' to the ZFN site in the same manner. The terminal number indicates the approximate size of deletion.

>d5-AAVS1-0.1kb
gggcccctatgtccacttcaggacagcatgtttgctgcctccagggatccCTAGGGACAGGATT*GGT*GACAGAAAA
GCCCCATCCTTAGGCCTCCTCCTT

>d3-AAVS1-0.1kb
aaggagagagatggctccaggaaatgggggtgtgtcaccagataaggaatCCCACTGTGGGGTGGAGGGGACAGAT
AAAAGTACCCAGAACCAGAGCCAC

>d5-AAVS1-0.5kb
tcctctccgggcatctctcctccctcacccaaccccatgccgtcttcactcgctgggttcCTAGGGACAGGATTGG
TGACAGAAAAGCCCCATCCTTAGG

>d5-AAVS1-1kb
cctggactttgtctccttccctgccctgccctctcctgaacctgagccagctcccatagcCTAGGGACAGGATTGG
TGACAGAAAAGCCCCATCCTTAGG

>d5-AAVS1-1.5kb
gccgaccctggccccggcgccgagctcgaccccgccgcgccgccgcccgcccgcgccgtgCTAGGGACAGGATTGG
TGACAGAAAAGCCCCATCCTTAGG

>d5-AAVS1-2kb
agctgcggacttcccagtgtgcatcggggcacagcgactcctggaagtggccaagggccaCTAGGGACAGGATTGG
TGACAGAAAAGCCCCATCCTTAGG

>d3-AAVS1-2kb
acgagggccaagagcatgaggtcatggaaactcgggctgtgaaggggccgcacgtgccctCCCACTGTGGGGTGGA
GGGGACAGATAAAAGTACCCAGAA

>d5-AAVS1-2.5kb
tacgctcgtcatcccagcaggattacaggccgaggcgggcggatcatgaggtcaagagatCTAGGGACAGGATTGG
TGACAGAAAAGCCCCATCCTTAGG

>d5-AAVS1-3kb
gagagccctgtccctgtggacactgtgtcttgggttcctttactcggtgtggctcagggaCTAGGGACAGGATTGG
TGACAGAAAAGCCCCATCCTTAGG

>d5-AAVS1-3.5kb
tcaccgcctagacattgagtcgccggatgtttcaatgcctcatgatacaataaaaccacaCTAGGGACAGGATTGG
TGACAGAAAAGCCCCATCCTTAGG

>d5-AAVS1-4kb
cacggaggtgttgtgcatgacgtctggacactagactagctgcatgtaatgggaaaagttCTAGGGACAGGATTGG
TGACAGAAAAGCCCCATCCTTAGG

>d5-AAVS1-4.5kb
ggtgtggtggctcatgcctgtaatcccagcactttgggaagctgaggcgggtggatcactCTAGGGACAGGATTGG
TGACAGAAAAGCCCCATCCTTAGG

>d5-AAVS1-5kb
ataagcaggtgaagttagaacatccacaaaagtataaaattgactcttctgtcctgtgtgCTAGGGACAGGATTGG
TGACAGAAAAGCCCCATCCTTAGG

>d5-AAVS1-10kb
gtcgcccaggctggagtgcagtggcacgatcttggctcactgcaagctctgcctcccaggCTAGGGACAGGATTGG
TGACAGAAAAGCCCCATCCTTAGG

>d5-AAVS1-10.1kb
aggagaatttgtaggcttcaagagaccatgttgtaacaggtgggtgataacaggctttaaCTAGGGACAGGATTGG
TGACAGAAAAGCCCCATCCTTAGG

>d5-AAVS1-19.9kb
tcaggagaccagcctgaccaatatgatgaaatcccatctctactaaaaatacaaaaattaCTAGGGACAGGATTGG
TGACAGAAAAGCCCCATCCTTAGG

>d5-AAVS1-20kb
ggccgggcgggcgcggcggctcacacctgtaatcccagcactttgggaggctgaggcgggCTAGGGACAGGATTGG
TGACAGAAAAGCCCCATCCTTAGG

>d5-AAVS1-50kb
tttggaactcctgggttctatagtggggaagagctcgaggatgaggtcttctgagtaatgCTAGGGACAGGATTGG
TGACAGAAAAGCCCCATCCTTAGG

>d5-AAVS1-100kb
acccaaagaagcttcttccactctgataaaagaaaggaaaaataggcaaagccacagacCTAGGGACAGGATTGGT
GACAGAAAAGCCCCATCCTTAGG

Supplementary Note 3. DNA sequences for ssODNs used to create deletions at various loci and a concerted 5 kb deletion + loxP insertion. Distal deletion sites are lowercase. The right or left ZFN binding sites are underlined. Bold uppercase letters indicate the 34 bp loxP site. d5 indicates a deletion 5' to the ZFN cut site (in the upstream direction on the sense strand of the gene sequence) and d3 indicates a deletion 3' to the ZFN site in the same manner. The terminal number indicates the approximate size of deletion.

>d5-IRAK4-5.9kb
gttcccaaaactgctaatacactaccttctaaagaagctataacagttcagcaaaaacagGAGATGCAAGATTGCT
CAGGGTGCAGCTAATGGCATCAAT

>d3-RSK2-5.2kb
agctcaaacataaaggaaaaaagtgtgtgtatgtacatatagagtggtaaaaagacttacTGTAGCTTTATGTATA
CATCTCTTGCAAACAGAGTAGGAG

>d3-RSK4-5kb
ttagaatgaatgttacagcttcccaaatgtgaaatctgtaccacagcctccccttcctgCAAACAGAGTAGGAGC
CAACACCAATATCCTCCTTCAATT

>d5-AAVS1-5kb-loxP
atccacaaaagtataaaattgactcttctgtcctgtgtg**ATAACTTCGTATAGCATACATTATACGAAGTTAT**CTA
GGGACAGGATTGGTGACAGAAAAGCCCCATCCTTA